Developmental Biology **219,** 154–162 (2000)
doi:10.1006/dbio.1999.9598, available online at http://www.idealibrary.com on IDEAL®

A 130-kDa Membrane Protein of Sperm Flagella Is the Receptor for Asterosaps, Sperm-Activating Peptides of Starfish *Asterias amurensis*

Takuya Nishigaki,[1] Kazuyoshi Chiba,[2] and Motonori Hoshi[3]

Department of Life Science, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8501, Japan

Spermatozoa of the starfish, *Asterias amurensis,* have a specific receptor for asterosap, a sperm-activating peptide isolated from the jelly coat of homologous eggs. We characterized the receptor by using several asterosap derivatives. Analysis of equilibrium binding of radioactive di-iodinated Bolton–Hunter reagent-labeled asterosap ($^{125}I_2$-BHP15) to the spermatozoa indicated that the cell has 1.1×10^5 binding sites of high affinity (K_d = 57 pM), and also the receptor showed positive cooperativity for asterosap binding. When spermatozoa were treated with fluorophore-labeled asterosap, the sperm flagella were labeled, indicating that the receptors are mostly localized in the sperm tail. When spermatozoa were reacted with radioactive asterosap prelabeled with photoaffinity cross-linkers, a single 130-kDa membrane protein of sperm flagella was specifically radiolabeled. This result was reproducible regardless of the length of spacer arm of cross-linkers so far studied. Therefore, the 130-kDa protein is likely to be the receptor for asterosaps. Modification of asterosap at the N-terminal region with bulky molecules such as carboxyfluorescein did not affect the activity of asterosap, suggesting that the N-terminus of asterosap is not involved in the ligand–receptor interaction. On the other hand, S-alkylated asterosaps did not compete with $^{125}I_2$-BHP15 for binding to the receptor, indicating that disulfide linkage of asterosap is essential for the ligand–receptor interaction. The properties of the receptor, high affinity and high concentration, enabled us to apply the fluorescence polarization technique to study the molecular interaction between asterosap and the receptor. Using this method, we performed binding experiments in almost real time and found that divalent cations are significantly involved in the interaction between asterosap and the receptor.

Key Words: **sperm-activating peptide; receptor; equilibrium binding; cross-linking; fluorescence polarization.**

INTRODUCTION

Sperm-activating peptide (SAP)[4] is one of the most characterized molecules involved in sperm–egg interaction. SAP is known as a diffusible component of the jelly coat of echinoderm eggs and is considered to play important roles in promoting efficient fertilization (Garbers, 1989). Since speract (SAP-1) was purified and structurally identified from sea urchins, *Hemicentrotus pulcherrimus* and *Strongylocentrotus purpuratus* (Suzuki *et al.*, 1981; Garbers *et al.*, 1982), more than 70 peptides have been identified in various species of echinoids (Suzuki and Yoshino, 1992). SAP was originally characterized as a factor that stimulates sperm motility and respiration in slightly acidic seawater (Ohtake, 1976). Now, SAP is considered to be a multifunctional peptide, attracting sperm to the egg (Ward *et al.*, 1985) and facilitating the acrosome reaction (Yamaguchi *et al.*, 1987).

Recently, 12 SAPs were purified and structurally identified from starfish, *Asterias amurensis,* and they were collectively named asterosaps (Nishigaki *et al.*, 1996). Asterosaps are glutamine-rich tetratriacontapeptides containing an intramolecular disulfide linkage between Cys^8 and Cys^{32}. They are much larger than sea urchin SAPs and do not share any structures with sea urchin analogues. Very recently, cDNAs encoding asterosap precursors have been

[1] Present address: Departamento Genética y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62250, México.

[2] Present address: Department of Biology, Ochanomizu University, 2-1-1 Ohtsuka, Tokyo 112-8610, Japan.

[3] To whom correspondence should be addressed. Fax: +81-45-924-5777. E-mail: mhoshi@bio.titech.ac.jp.

[4] Abbreviations used: AR, acrosome reaction; ARIS, acrosome reaction-inducing substance; ASW, artificial seawater; BHR, Bolton–Hunter reagent, *N*-succinimidyl-3-(4-hydroxyphenyl)propionate; CBB, Coomassie brilliant blue; DMSO, dimethyl sulfoxide; DW, deionized water; EDTA, ethylenediaminetetraacetic acid; NHS-ASA, *N*-hydroxysuccinimidyl-4-azidosalicylic acid; NHS-fluorescein, 5-carboxyfluorescein succinimidyl ester; SAP, sperm-activating peptide; SASD, sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-1-3′-dithiopropionate; TFA, trifluoroacetic acid; nAChR, nicotinic acetylcholine receptor; $^{125}I_2$-BHP15, radioactive di-iodinated Bolton–Hunter reagent-labeled asterosap; I-BHP15, monoiodinated BH-asterosap; F-P15, NHS-fluorescein-labeled asterosap.

0012-1606/00 $35.00

cloned from homologous ovaries (Matsumoto *et al.*, 1999). The sequence of the clones revealed that asterosap precursors contain multiple isoforms of asterosap like speract precursors. While speract is synthesized in the follicle cells (Kinoh *et al.*, 1994), asterosap is demonstrated to be synthesized in the oocytes by *in situ* hybridization. This difference in the site of synthesis also implies that sea urchin SAPs and asterosaps are not directly related in evolution even though their overall gene structures and functions are quite similar.

Starfish spermatozoa undergo the acrosome reaction upon encountering the jelly coat of homologous eggs (Dale *et al.*, 1981; Ikadai and Hoshi, 1981). In *A. amurensis*, acrosome reaction is induced by the action in concert with three components in the jelly coat (Hoshi *et al.*, 1994): (1) a very large, sulfated glycoprotein named acrosome reaction-inducing substance (ARIS), of which the structure of a bioactive major polysaccharide chain has recently been identified (Koyota *et al.*, 1997); (2) sulfated steroid saponins named Co-ARIS; and (3) asterosaps. Experimentally, ARIS is capable of inducing the acrosome reaction in high-pH or high-Ca^{2+} seawater, while ARIS and Co-ARIS or ARIS and asterosap are required in normal seawater. The anti-asterosap antibody neutralized the ability of the egg jelly as the inducer of acrosome reaction, and the addition of excess asterosap recovered the neutralization (Nishigaki *et al.*, 1996). Therefore, asterosap is physiologically essential for the induction of acrosome reaction in starfish.

In this study, we characterized the receptor for asterosap in starfish spermatozoa to clarify the mechanism of sperm activation and to understand its involvement in the induction of acrosome reaction in starfish.

MATERIALS AND METHODS

Materials. Starfish, *A. amurensis*, were collected from several locations in Japan. The starfish in Tasmania, Australia, were used in some experiments. No significant differences were found between Japanese and Tasmanian animals (Byrne *et al.*, 1997). Sperm were obtained "dry" by cutting the testes and kept on ice before use (Matsui *et al.*, 1986). Artificial seawater (ASW) consisted of 430 mM NaCl, 9 mM $CaCl_2$, 9 mM KCl, 23 mM $MgCl_2$, 25 mM $MgSO_4$, and 10 mM EPPS (pH 8.2 with NaOH). 5-Carboxyfluorescein succinimidyl ester (NHS-fluorescein) was purchased from Takara Co. Bolton–Hunter reagent, NHS-rhodamine, and photoaffinity cross-linkers were purchased from Pierce Co. Deoxyribonuclease I (DNase I) from bovine pancreas was purchased from Sigma Co. Synthesized asterosaps [P15; Gly-Gln-Thr-Gln-Phe-Gly-Val-Cys*-Ile-Ala-Arg-Val-Arg-Gln-Gln-His-Gln-Gly-Gln-Asp-Glu-Ala-Ser-Ile-Phe-Gln-Ala-Ile-Leu-Ser-Gln-Cys*-Gln-Ser, intramolecular disulfide linkage is formed between two Cys* and P9-1; (Asp^2, Lys^3, Glu^4, Met^5)P15] were kindly provided by Drs. Miki and Minakata, Suntory Institute for Fundamental Research.

Iodination of Bolton–Hunter reagent (BHR) with nonradioactive sodium iodide. Twenty microliters of NaI solution (3 mg/ml for di-iodinated form) was mixed with 2 μl of BHR (5 mg/ml in DMSO). The reaction was initiated by addition of 10 μl of chloramine-T (5 mg/ml) to the solution on ice and, 10 s after initiation, terminated by the addition of 10 μl of $Na_2S_2O_5$ (12 mg/ml). After addition of 5 μl DMF to the solution, both reacted and nonreacted BHRs were extracted with 200 μl of benzene twice and concentrated by N_2 stream. Sodium iodide, chloramine-T, and $Na_2S_2O_5$ were dissolved in 250 mM sodium phosphate buffer, pH 7.5. To obtain monoiodinated BHR (mixture with non- and di-iodinated BHRs), the amount of NaI for the reaction was decreased.

Labeling asterosap with Bolton–Hunter reagents (non-, mono-, and di-iodinated forms). A synthesized asterosap (P15; 2–10 nmol) was dissolved in 100 μl of 100 mM sodium borate buffer at pH 8.5, and about fivefold of BHR (in DMSO) was added to the solution and mixed well for 1 h at room temperature. The excess reagents were blocked by addition of 10 μl of 1 M glycine to the solution and incubated for 15 min at room temperature. The labeled peptides (BHP15, I-BHP15, and I_2-BHP15) were purified by reverse-phase HPLC.

Purification of asterosap derivatives by reverse-phase HPLC. HPLC was performed on a C_4 column (Cosmosil 5C4-300, 4.6 $\times$ 150 or 4.6 $\times$ 10 mm; Nacalai Tesque Co.). The column eluate was monitored by absorbance at 220 nm. The column was equilibrated with 20% acetonitrile in 0.1% trifluoroacetic acid (TFA) followed by a linear gradient up to 50% acetonitrile in 0.1% TFA for 30 min at a flow rate of 1 ml/min. Elution times of P15, BHP15, I-BHP15, and I_2-BHP15 using the small column (4.6 $\times$ 10 mm) are 12, 13.5, 15, and 16 min, respectively.

Measurement of sperm motility. Sperm motility was measured by a video tape recording analysis in slightly acidified ASW, pH 6.4 (Nishigaki *et al.*, 1996).

Preparation of radioactive di-iodinated Bolton–Hunter reagent-labeled asterosap ($^{125}I_2$-BHP15). One grain of Iodo-Beads (Pierce Co) was put in 100 μl of 200 mM phosphate buffer at pH 7.0, and $Na^{125}I$ (3.7 MBq) solution was mixed and incubated for 5 min. Monoiodinated BH-asterosap (I-BHP15, 2 nmol) was added to the solution and incubated for 5 min, and 10 μl of nonradioactive NaI (100 mg/ml) was added to the solution to terminate the reaction. The solution was applied to a cartridge of Sep-Pak light tC_{18} and washed with 2 ml of NaI (100 mg/ml) solution, 5 ml of DW, and 5 ml of 20% MeOH, and $^{125}I_2$-BHP15 was eluted with 0.5 ml of 80% MeOH. The eluate was concentrated by N_2 stream and applied to reverse-phase HPLC further purification.

Binding experiments by centrifugation assay. $^{125}I_2$-BHP15 was mixed with an appropriate amount of nonradioactive I_2-BHP15 to use an accurate concentration of radioactive ligand for binding experiments. The specific activity of the $^{125}I_2$-BHP15 was approximately 1 kBq/pmol. Dry sperm was diluted 4000-fold and incubated with $^{125}I_2$-BHP15 for 5 min at 23°C in 200 μl of ASW (pH 8.2) containing 0.1% BSA in a microfuge tube. The tube was centrifuged for 1 min at 2000*g* at 4°C and 100 μl of supernatant was immediately transferred to another tube. Radioactivity in both 100 μl of supernatant corresponding to the half of free ligand and the rest of the fraction, which corresponds to another half of free ligand and total bound ligand, was measured by a γ counter (Aloka Auto Well Gamma System ARC-300), and concentrations of bound and free ligand were calculated. Various concentrations of $^{125}I_2$-BHP15 were prepared by serial dilution (from 32 nM to pM range). Nonspecific binding was determined by incubation of spermatozoa in the presence of 1 μM of a synthesized asterosap (P15 or P9-1). The number of spermatozoa was counted using a hemocytometer to obtain the number of binding sites in a single cell.

Labeling asterosap with fluorophore. NHS-fluorescein were used for labeling asterosap (P15) with fluorophore. Each labeling was performed similarly as labeling with Bolton–Hunter reagents.

Fluorescence microscopy. Dry sperm was diluted 200-fold and incubated with 100 nM NHS-fluorescein-labeled asterosap (F-P15)

for 5 min in 20 μl of ASW at pH 8.2. Spermatozoa were fixed by adding 1 μl of 10% formaldehyde, and 5 μl of the suspension was dropped onto a polylysine-coated glass slide. The cells were rinsed with ASW containing 0.01% Tween 20 and covered with a coverslip. Photobleaching of F-15 was very fast; thus propylgarate (1% at final concentration) was added to the cell. Nonspecific binding was determined by incubation of spermatozoa in the presence of 10 μM of asterosap (P15). Micrographs were taken at ASA 1600. G L.

Labeling asterosap with photoaffinity cross-linkers. SASD and NHS-ASA were used for labeling asterosap (P15) with photoaffinity cross-linkers. Labeling (in the dark) was performed similarly to that with Bolton–Hunter reagent. A peptide deleted of the N-terminal five residues (dN5-asterosap) by cyanogen bromide oxidation of P9-1 (Nishigaki *et al.*, 1996) was also labeled by NHS-ASA. All labeled peptides were purified by reverse-phase HPLC. The spacer arm of SASD and NHS-ASA is 18.9 and 8.0 Å, respectively.

Iodination of cross-linker-labeled asterosap with radioactive sodium iodide. The reaction was carried out in almost the same way as described under "Preparation of $^{125}I_2$-BHP15." SASD-labeled asterosap (1.5 nmol), NHS-ASA-labeled asterosap (1.8 nmol), and NHS-ASA-labeled dN5-asterosap (0.4 nmol) were used for iodination. For cross-linking experiments, 80% MeOH eluate from a cartridge of Sep-Pak light tC_{18} was used.

Preparation of sperm membrane. Dry sperm (also sperm head and sperm tail described below) was diluted fivefold with a cavitation buffer containing 200 mM KCl, 1.25 mM $MgCl_2$, 400 mM mannitol, 2 mM benzamidine, 0.5 mM phenylmethane sulfonyl fluoride, 25 mM Hepes, and 25 mM Pipes (pH 6.8 with KOH). The sperm suspension was subjected to nitrogen cavitation in a Parr nitrogen cavitation bomb for 30 min at 1000 psi with stirring on ice. The resultant suspension was centrifuged at 10,000*g* for 1 h. The supernatant was centrifuged at 100,000*g* for 30 min. The pellet was resuspended in the same buffer at a protein concentration of 6.8 mg/ml (determined by BCA method using BSA as a standard). All procedures were carried out at 0–4°C. The sperm membrane was stored at −80°C.

Separation of heads and tails of spermatozoa. Dry sperm was diluted with 10 vol of ASW. The sperm suspension was passed three times through a 22-gauge needle (45 mm in length) on ice and centrifuged at 1000*g* for 5 min at 4°C to precipitate the sperm heads. Sperm tails were recovered from the supernatant by centrifugation at 10,000*g* for 20 min at 4°C. Sperm heads and tails were washed and resuspended in ASW (10 vol of dry sperm).

Cross-linking experiments. Dry sperm was diluted 40-fold and incubated with radioactive cross-linker-labeled asterosaps in 20 μl of ASW (pH 8.2) for 10 min on ice. The amount of the peptides and their radioactivity were estimated as follows; ^{125}I-SASD-labeled asterosap, 5 pmol and 1.2 kBq; ^{125}I-NHS-ASA-labeled asterosap, 6 pmol and 3 kBq; and ^{125}I-NHS-ASA-labeled dN5-asterosap, 2.2 pmol and 1.6 kBq. The sperm suspension was photoactivated with UV light (365 nm) for 10 min on ice and mixed with 20 μl of 2× SDS–PAGE sample buffer and then immediately boiled for 5 min. Prior to addition of SDS–PAGE sample buffer, the sperm suspension was mixed well with 1 μl of DNase I (1 mg/ml in DW) to reduce viscosity. Sperm membrane (1.7 mg protein/ml at final) was cross-linked with the same procedure except DNase treatment was omitted. Each sample of 5 μl was subjected to 10% SDS–PAGE, and protein bands were stained with CBB. Imaging of radioactivity was performed by a BAS 2000 Fuji Imaging system.

S-alkylation of asterosap. S-pyridylethylation of asterosap was carried out according to the methods previously described (Nishigaki *et al.*, 1996). S-carboxymethylation and S-carboxyamidomethylation of asterosap were carried out as follows: about 10 nmol of synthesized asterosap P15 was dissolved in 100 μl of 1 mg/ml of dithiothreitol, 7 M guanidine hydrochloride, 10 mM EDTA, and 0.5 M Tris–HCl at pH 8.5 and incubated over 4 h at room temperature in the dark while stirring. For alkylation, 10 μl of 25 mg/ml iodoacetic acid or iodoacetamide was added to the reaction mixture and incubated an additional 30 min at room temperature in the dark while stirring. The reacted samples were purified with reverse-phase HPLC. Purified S-alkylated asterosaps were dissolved in DMSO and used for competition experiments.

Binding experiments by fluorescence polarization. NHS-fluorescein-labeled asterosap (F-P15), which was used for fluorescence microscopy, was added to 1 ml ASW at 20°C in a glass tube; then sperm or sperm membrane was added to the tube. Fluorescence polarization (*P*) was automatically measured by BEACON (PanVera Co.). *P* is defined as follows: $P = (Int \parallel - Int \perp)/(Int \parallel + Int \perp)$, where Int || is the intensity of the emission light parallel to the excitation light plane and Int ⊥ is the intensity of the emission light perpendicular to the excitation light plane (Walter *et al.*, 1981). *P*, being a ratio of light intensities, is a dimensionless number and has a maximum value of 0.5 for fluorescein. Fluorophore-labeled ligand without the receptor (free ligand) show low *P*, while the ligand bound to the receptor gives high *P*. This characteristic provides a bound/free ratio of ligand without any physical separation of bound and free ligand independent of the absolute concentrations of the ligand. Fluorescence polarization (*mP*) can be measured every 13 s by BEACON.

Effects of cations on asterosap–receptor interaction. F-asterosap and sperm membrane (or sperm) was incubated in divalent cation-free ASW containing 5 mM EDTA for a few minutes. One molar solution of metal chlorides ($CaCl_2$, $CdCl_2$, $CoCl_2$, KCl, LiCl, $MgCl_2$, $MnCl_2$, NaCl, $NiCl_2$, or RbCl) was added to the suspension to test the effect of each action on asterosap–receptor binding.

RESULTS

Binding experiments using a radioactive asterosap derivative. As asterosaps do not have any tyrosine residues, which is required for iodination, asterosap was modified with BHR for radiolabeling. Since an asterosap P15 does not have any lysine residues, BHR reacted only to amino-terminal amine (α-NH_2) of the asterosap. The di-iodinated form of the peptide analogues was purified by reverse-phase HPLC. It accelerated sperm motility in slightly acidified ASW as intact asterosap (P15) did (Fig. 1a). Thus, the introduction of the large iodinated group in the amino terminus of asterosap did not appear to affect the biological activity, which is also supported by a result of competition assay described later (Fig. 6).

First, the time course of ligand association with sperm was determined to establish the time needed for equilibrium. Binding experiments were carried out by centrifugation binding assay as described under Materials and Methods. Spermatozoa were incubated with $^{125}I_2$-BHP15 for various periods as shown in Fig. 1b. The data showed that the ligand was very rapidly bound to sperm. Thus, subsequent binding experiments were performed incubating spermatozoa with $^{125}I_2$-BHP15 for 5 min.

Next, spermatozoa were incubated with various concentrations of $^{125}I_2$-BHP15 and the bound and free concentra-

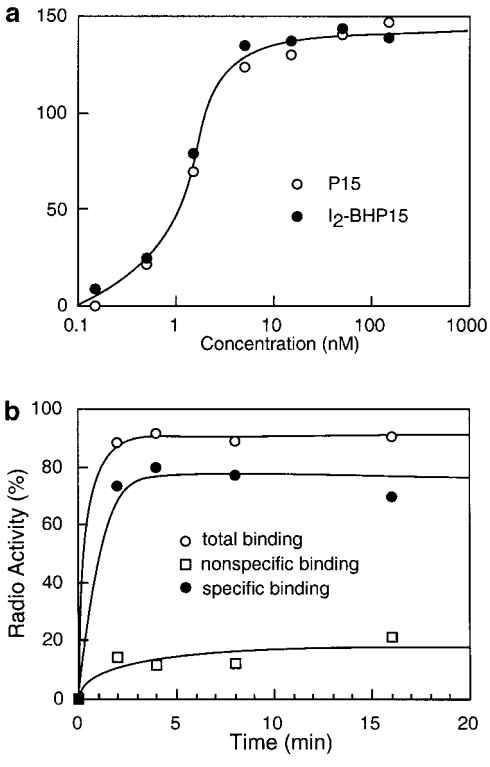

FIG. 1. (a) Biological activity of di-iodinated Bolton–Hunter-labeled asterosap (I_2-BHP15). Sperm motility was measured in slightly acidified ASW as described under Materials and Methods. Each point is the mean of two experiments. (b) Time course of $^{125}I_2$-BHP15 binding to the sperm. One nM $^{125}I_2$-BHP15 and 1000-fold diluted dry sperm were incubated in a microfuge tube. Bound ligands were separated from free ligands by centrifugation at the indicated time. Nonspecific binding was measured in the presence of 1 μM asterosap (P9-1).

tions of the ligand were measured as described under Materials and Methods. Plotting the data with a linear abscissa (Fig. 2a), specific binding of $^{125}I_2$-BHP15 to spermatozoa was demonstrated to be saturable. Interestingly, a Scatchard plot of the data yielded an upward convex (Fig. 2b), suggesting the occurrence of positive cooperativity between binding sites (Scatchard, 1949; Dahlquist, 1978). The data were also plotted with a logarithmic abscissa according to the method of Klotz (1982). As a result, a single sigmoidal curve was yielded (Fig. 2c), and the dissociation constant (K_d) and total receptor concentrations (B_{max}) were estimated to be 57 pM and 1.1 $\times$ 10^5 sites/spermatozoon, respectively.

Receptor localization examined by fluorescence microscopy. Localization of asterosap receptors on sperm was determined using fluorescence microscopy and fluorophore-labeled asterosap. F-P15 competed with $^{125}I_2$-BHP15 for binding to the receptor in almost the same manner as P15 (Fig. 6a). Thus, fluorescent tag did not appear to affect the biological activity. Figure 3 shows that F-P15 bound almost exclusively to the flagella of spermatozoa. These signals were not observed in the presence of excess intact asterosap (data not shown). Therefore, asterosap receptors seem to be localized mainly on the sperm flagella.

Cross-linking experiments using photoaffinity cross-linker. Photoaffinity cross-linker SASD and NHS-ASA were used for cross-linking experiments. To produce a cross-linking probe with a short actual spacer arm, dN5-asterosap (asterosap deleted N-terminal 5 residues of P9-1) was prepared additionally and labeled with NHS-ASA. Figure 4 shows that a 130-kDa protein, which is a major sperm membrane protein, was specifically cross-reacted with all asterosap derivatives in both intact sperm and

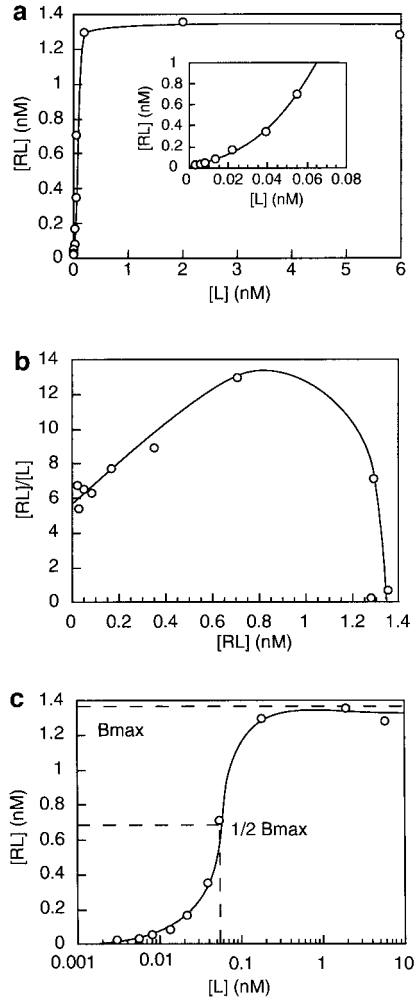

FIG. 2. Equilibrium binding of radioactive di-iodinated Bolton–Hunter reagent-labeled asterosap ($^{125}I_2$-BHP15). (a) Saturation curve of $^{125}I_2$-BHP15 binding. The specific binding was plotted against free ligand ($^{125}I_2$-BHP15) with a linear abscissa. Each point is the mean of three experiments. (b) Scatchard plot. The molar ratio of specifically bound ligand ($^{125}I_2$-BHP15) to free ligand was plotted against bound ligands. (c) Klotz plot. The specific binding was plotted against free ligand ($^{125}I_2$-BHP15) with a logarithmic abscissa.

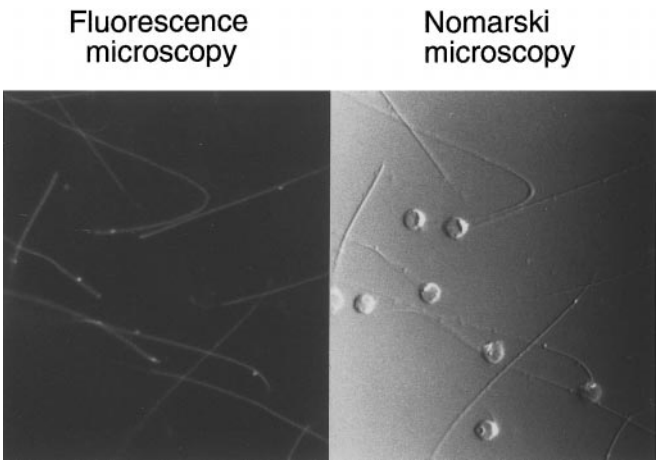

FIG. 3. Fluorescence microscopy. The spermatozoa incubated with fluorophore-labeled asterosaps. NHS-fluorescein-labeled asterosap was incubated with spermatozoa as described under Materials and Methods.

sperm membrane. Although radioactivity of the band is higher under nonreducing conditions with an unknown reason, there was no mobility change of this protein in SDS–PAGE under this condition, indicating that this protein does not exist as multimer formed by disulfide linkage.

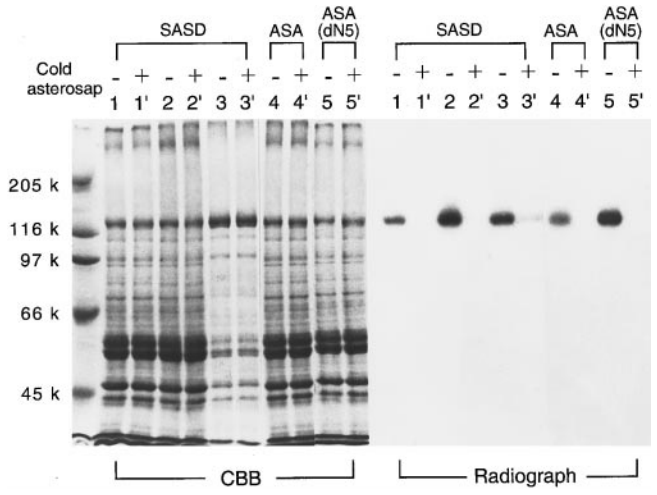

FIG. 4. Photoaffinity cross-linking of three asterosap analogues. CBB staining (left) and radioactivity imaging (right) of SDS–PAGE (10%) of sperm and sperm membrane are shown. Loaded samples: intact sperm and ^{125}I-SASD-labeled asterosap (lane 1); same as lane 1 under nonreducing condition (lane 2); sperm membrane and ^{125}I-SASD-labeled asterosap (lane 3); intact sperm and ^{125}I-NHS-ASA-labeled asterosap (lane 4); intact sperm and ^{125}I-NHS-ASA-labeled dN5-asterosap (lane 5). Cross-linking was performed in the presence of 5 μM cold asterosap P15 in each sample (lanes 1′, 2′, 3′, 4′, and 5′).

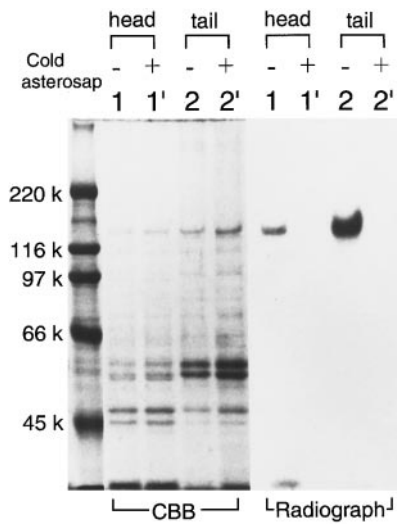

FIG. 5. Photoaffinity cross-linking of an asterosap analogue to sperm heads and tails. CBB staining (left) and radioactivity imaging (right) of SDS–PAGE (10%) of sperm and sperm membrane are shown. Loaded samples: sperm head and ^{125}I-SASD-labeled asterosap (lane 1); sperm tail and ^{125}I-SASD-labeled asterosap (lane 2). Cross-linking was also performed in the presence of 5 μM cold asterosap in each sample (lanes 1′ and 2′).

When a cross-linking experiment was performed with the sperm head or the sperm tail, the signal of a 130-kDa protein was found mainly in the sperm tail (Fig. 5), which corresponds to the previous result (Fig. 3). Thus, we conclude that a 130-kDa protein is a receptor of asterosap.

Molecular interaction of S-alkylated asterosaps and the receptor. A disulfide linkage of asterosap is known to be important for its activity (Nishigaki *et al.*, 1996). To investigate effects of S-alkylation of asterosap on ligand–receptor interaction, competition assays were carried out using three S-alkylated (S-pyridylethylated, S-carboxyamidomethylated, and S-carboxymethylated) P15 analogues. Figure 6b clearly shows that all S-alkylated asterosap analogues did not compete with $^{125}I_2$-BHP15. These results indicate that the disulfide linkage of asterosap is essential for molecular interaction between asterosap and the receptor. In contrast, P15, P9-1, and nonradioactive I_2-BHP15 dose-dependently decreased $^{125}I_2$-BHP15 binding to the spermatozoa in the same manner. These results support the conclusion that amino-terminus of asterosap is not important for the activity of asterosap.

Binding experiments using fluorescence polarization system, BEACON. Binding experiment was also carried out using a fluorescence polarization system. Figure 7a demonstrates the relationship between bound ligand and sperm concentration. The fluorescence polarization method gave a similar result as the centrifugation method in this experiment. Thus, the fluorescence polarization system seems applicable to the study of asterosap–receptor interactions. However, we found F-P15 slightly quenched

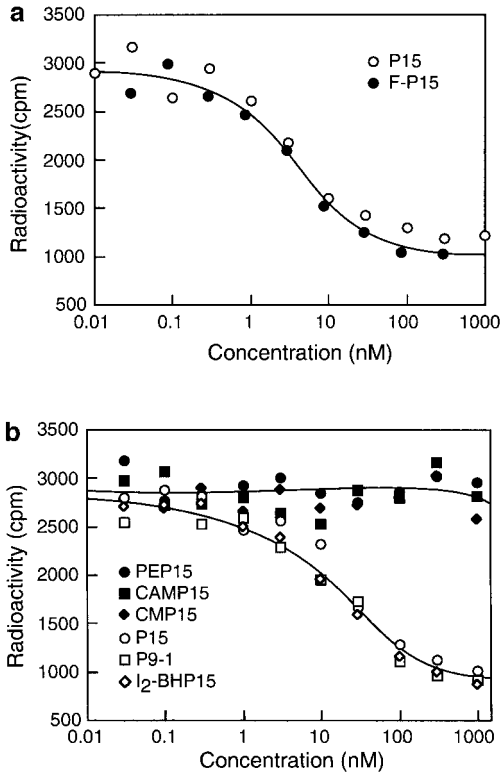

FIG. 6. Competition binding assay. Binding experiments were carried out using 1 nM radioactive ligand ($^{125}I_2$-BHP15) by centrifugation assay. The radioactivity of total binding was plotted against the concentration of competitors. (a) Competition assays by fluorophore-labeled asterosaps. Competitors: unlabeled asterosap (P15) and NHS-fluorescein-labeled asterosap (F-P15). Each point is the mean of three experiments. (b) Competition assays of S-alkylated asterosaps. Competitors: intact asterosap (P15, P9-1), nonradioactive di-iodinated Bolton–Hunter reagent-labeled asterosap (I_2-BHP15), S-pyridylethylated asterosap (PEP15), S-carboxyamidomethylated asterosap (CAMP15), and S-carboxymethylated asterosap (CMP15). Each point is the mean of three experiments.

upon the receptor binding (data not shown), indicating that fluorescence polarization value loses the proportional relationship with bound/free ratio of ligand (Walter *et al.*, 1981). Thus, it is required to determine the exact ratio of the quenching in order to utilize the fluorescence polarization system for the quantitative study of asterosap–receptor interactions.

The time course of F-P15 binding to sperm was measured using the fluorescence polarization system. Figure 7b shows that F-P15 was rapidly associated with the receptor, and also the ligand was rapidly dissociated from the receptor when an excess amount of unlabeled asterosap was added.

When sperm membrane was incubated in 20 mM Tris–HCl buffer (pH 7.4 containing 150 mM NaCl) without divalent cations, ligand-binding activity of the membrane was remarkably decreased, and the activity was recovered by the addition of $CaCl_2$ or $MgCl_2$ (not NaCl nor KCl) solution into the buffer (data not shown), suggesting that divalent cations are involved in asterosap–receptor interaction. To investigate the effects of divalent cations on asterosap–receptor interaction, sperm membrane was incubated in divalent cation-free ASW containing 5 mM EDTA, and then the various metal chloride solution was added to the ASW. Figure 8 shows that polarization (*P*) did not increase to the normal level when sperm membrane was incubated in divalent cation free ASW. The *P* value increased dependent upon the concentration of Ca^{2+}, whereas it did not increase by addition of K^+. Other divalent cations tested, Cd^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, and Ni^{2+}, showed similar effects as Ca^{2+} upon *P* value, and monovalent cations tested

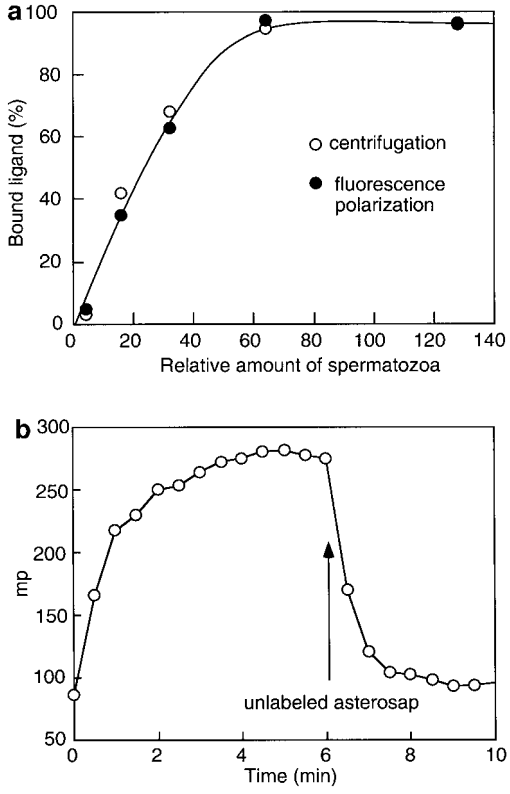

FIG. 7. (a) Comparison between centrifugation and fluorescence polarization in binding experiments. After incubating 1 nM NHS-fluorescein-labeled asterosap (F-P15) with various concentrations of sperm, percentages of bound F-P15 were determined by centrifugation and fluorescence polarization as described under Materials and Methods. In fluorescence polarization, it was assumed that all ligands are free when *mP* is 100; all ligands are bound when *mP* is 410. Hence, percentages of bound ligands was calculated as follows: Bound % = (observed *mP* − 100)/(410 − 100) × 100. (b) Time course of the binding of NHS-fluorescein-labeled asterosap (F-P15) to spermatozoa utilizing fluorescence polarization. F-P15 (1 nM) was incubated in 1 ml ASW at 20°C in a glass tube and then 5 μl of 100-fold diluted dry sperm was added to the tube. After 6 min, 10 μl of 10 μM (100 nM at final) unlabeled P15 was added to the tube. Fluorescence polarization was measured by BEACON every 30 s. The data are representative of several experiments.

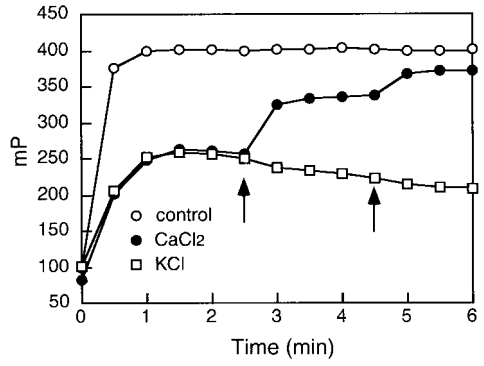

FIG. 8. Effect of divalent cation on asterosap–receptor interaction. F-P15 (1 nM) was incubated in 1 ml normal ASW (open circle) or divalent-cation-free ASW containing 5 mM EDTA (all other symbols) at 20°C in a glass tube, and then 5 μl of 5 mg protein/ml sperm membrane was added to each tube. After 2.5 and 4.5 min (indicated with arrows), 10 μl of 1 M metal chloride solution (10 mM at final) was added to divalent cation free ASW. Fluorescence polarization (*mP*) was measured by BEACON every 30 s in the course of experiments. The data are representative of several experiments.

(Li^+ and Rb^+) have no positive effects upon *P* (data not shown). In the absence of sperm membrane, divalent cations did not increase *P* of F-P15, and the effect of divalent cations on the ligand–receptor interaction was confirmed by ultracentrifugation. Thus, the effect of divalent cations was not caused by experimental artifacts. Similar results were obtained using intact spermatozoa instead of sperm membrane. Therefore, divalent cations are significantly important for asterosap–receptor interaction.

DISCUSSION

The N-terminal region of asterosap is not important for the biological activity of this peptide (Nishigaki *et al.*, 1996). This property enabled us to label asterosaps at this region with radioisotope, fluorophores, and photoaffinity cross-linkers without any negative effects on their bioactivity, which led to successful characterization of the receptor on sperm plasma membrane.

Equilibrium binding experiments. We first performed the equilibrium binding experiments using radioisotope-labeled asterosap by centrifugation assay. Starfish spermatozoa appeared to have only one type of receptor for asterosap since Klotz plots yielded a single sigmoidal curve (Fig. 2c). The dissociation constant (K_d) of the starfish receptor was very low (57 pM), and maximum binding sites (B_{max}) on the sperm surface was very large (1.1×10^5/cell). B_{max} determined in this study is much larger than that of speract receptor of *S. purpuratus*, 6000–8000 (Smith and Garbers, 1983), but not so different from that of SAPIII receptor of *Clypeaster japonicus*, 3.4×10^4 for high affinity and 6.1×10^4 for low affinity (Yoshino and Suzuki, 1992). Assuming that the sperm flagellum has about 50-μm^2 surface area (0.3 μm in diameter, 50 μm in length), the B_{max} of asterosap receptor would represent about $2 \times 10^3/\mu m^2$. Nicotinic acetylcholine receptor (nAChR) in postsynaptic membranes of electric tissue of electric rays was reported to exist at high density, about $1 \times 10^4/\mu m^2$ (Heuser and Salpeter, 1979), which is known as one of the most densely localized receptors. Considering that the molecular weight of nAChR (290 kDa) is much higher than that of asterosap receptor (130 kDa), the plasma membrane of sperm tail has enough space for the large number of asterosap receptor determined in this study.

The asterosap receptor showed positive cooperativity (Fig. 2b) like sea urchin receptors (Yoshino and Suzuki, 1992; Shimizu *et al.*, 1994), suggesting that this is a common characteristic among SAP receptors although the physiological meaning is unknown. Divalent cations may play a role for this positive cooperativity as discussed later.

Fluorescence microscopy. Localization of the receptor for asterosap in sperm was studied using F-P15. Figure 3 shows that F-P15 bound almost exclusively to the flagella of spermatozoa, indicating that the receptors for asterosap are mostly localized on the surface of sperm flagella. The same result was obtained when rhodamine was used as fluorophore instead of fluorescein (data not shown). Such distribution of the speract receptor was reported in sea urchin spermatozoa (Cardullo *et al.*, 1994). Acrosome reaction (AR) is an event of exocytosis occurring in the head region of sperm, and asterosap is involved in the induction of AR (Nishigaki *et al.*, 1996). Thus, the asterosap receptor on the sperm tail is likely to be involved in the induction of AR, although a possible minor population of asterosap receptor on the sperm head may also play a role in the induction of AR. Therefore, further study is necessary to determine what kind of signals will extend to the head when asterosap is bound to the receptor. Visualization of intracellular pH and intracellular Ca^{2+} concentration of a single starfish sperm (*Asterina pectinifera*) was reported (Sase *et al.*, 1995). Similar methods should provide part, if not all, of the answer to this inquiry.

Cross-linking. Figure 4 shows that only a 130-kDa protein specifically cross-reacted with all asterosap derivatives. This protein is a major protein of the sperm membrane proteins, and this protein band is visible when intact sperm was applied to SDS–PAGE and stained with CBB. It accounts for the enormous number of receptors on the sperm plasma membrane. Therefore, the 130-kDa protein is most likely to be the asterosap receptor. The cross-reactions with the 130-kDa protein were found mainly in the sperm tail (Fig. 5), which corresponds to the localization of the receptor studied by fluorescent microscopy. We could not determine whether a small signal seen in the head was due to contamination of sperm tail. The possibility of small population of the receptor in the head cannot be excluded as described above.

Very recently, we cloned a homologue of guanylate cyclase from the starfish testis. The deduced N-terminal

amino acid sequence of the clone was consistent with N-terminal sequence of the cross-linked protein (manuscript in preparation). Thus, we conclude the 130-kDa protein cross-reacted with asterosap analogues is membrane-bound guanylate cyclase. Resact (SAP-IIA) receptor, 160 kDa from *Arbacia punctulata,* was reported to be guanylate cyclase (Shimomura *et al.*, 1986; Singh *et al.*, 1988) and SAP-I receptors, 77 kDa from *S. purpuratus* (Dangott and Garbers, 1984; Dangott *et al.*, 1989) and 71 kDa from *H. pulcherrimus* (Shimizu *et al.*, 1994), are believed to be coupled onto membrane-bound guanylate cyclase. Therefore, guanylate cyclase seems ubiquitously involved in a signal cascade of SAP.

Structure–function relationship of asterosap on ligand–receptor interaction. The structure–function relationship of asterosap can be discussed at the point of molecular interaction between asterosap and the receptor. When the disulfide linkage of asterosap was reduced and S-alkylated with several reagents, the affinity of the asterosap analogues to the receptor significantly decreased (Fig. 6b). This result can account for loss of the activity of S-pyridylethylated asterosap on sperm motility (Nishigaki *et al.*, 1996) and indicates that disulfide linkage is essential for ligand–receptor interaction of asterosap. In contrast, modification of asterosap at its N-terminal amine with bulky molecules such as carboxyfluorescein did not affect the binding to the receptor (Fig. 6a). This result indicates that N-terminal region of asterosap is not involved in molecular interaction between asterosap and the receptor.

Fluorescence polarization. The fluorescence polarization system, BEACON, is an excellent system to study kinetics of molecular interactions because it provides a bound/free ratio of a fluorophore-labeled ligand without any mechanical separation as described under Materials and Methods. However, this method is not always applicable to study ligand–receptor interactions because it is often difficult to prepare cells or membrane vesicles that contain enough concentration of the receptor to obtain reliable data. As mentioned above, starfish spermatozoa have an enormous number (1.1×10^5/cell) of high-affinity (K_d = 57 pM) receptors on their plasma membrane, so application of the fluorescence polarization for the binding experiment of asterosap was successful. We could rapidly and continuously monitor asterosap–receptor interaction using F-P15. We demonstrated that F-P15 rapidly associated with and dissociated from the receptors (Fig. 7b). Furthermore, we found that divalent cations are significantly involved in asterosap–receptor interaction (Fig. 8). Smith and Garbers (1983) reported that the ionic composition of the seawater greatly affects binding of speract to the receptor, and they demonstrated Na^+ and Mg^{2+} in seawater are important for speract–receptor interaction. It is established that SAPs change ionic permeability across the sperm plasma membrane and alter the cellular metabolism such as membrane potential, intracellular pH, and intracellular Ca^{2+} concentration (Darszon *et al.*, 1999). Thus, it is worth studying the effect of changing ionic environment on the receptor function from the point of view of positive or negative feedback regulation of the receptor.

The fluorescence polarization technique was shown to be an excellent tool to study molecular interactions between asterosap and the receptor. However, quenching of F-P15 upon the receptor binding (around 25%, data not shown) limits its usefulness as mentioned under Results. Therefore, determination of the quenching ratio is necessary to apply this method to quantitative measurements (kinetic studies) of asterosap–receptor interactions. Studies on the receptor using this method may lead to further understanding of the regulatory mechanism of SAP receptor.

ACKNOWLEDGMENTS

We greatly appreciate Dr. W. Miki and Dr. H. Minakata, Suntory Institute for Fundamental Research, for providing synthetic peptides. We thank the directors and staff of Otsuchi Marine Research Center and Misaki Marine Biological Station, University of Tokyo; Akkeshi Marine Biological Station, Hokkaido University; Dr. M. Komatsu of Toyama University; and Dr. M. Byrne of University of Sydney, for their help in collecting starfish. We thank D. D. Griffin for her review of the manuscript. T.N. thanks JSPS Research Fellowships for Young Scientists for generous financial assistance. This study was supported in part by Grants-in-Aid for Scientific Research (No. 08458237) and for Joint Research, International Scientific Research Program (No. 09044210) by Ministry of Education, Science, Sports and Culture, Japan, and by the Cooperative Program, Ocean Reasearch Institute, University of Tokyo.

REFERENCES

Byrne, M., Morrice, M. G., and Wolf, B. (1997). Introduction of the Northern Pacific asteroid *Asterias amurensis* to Tasmania—Reproduction and current distribution. *Marine Biol.* **127,** 673–685.

Cardullo, R. A., Herrick, S. B., Peterson, M. J., and Dangott, L. J. (1994). Speract receptors are localized on sea urchin flagella using a fluorescent peptide analog. *Dev. Biol.* **162,** 600–607.

Dahlquist, F. W. (1978). The meaning of Scatchard and Hill plots. *Methods Enzymol.* **48,** 270–299.

Dale, B., Dan-Sohkawa, M., De Santis, A., and Hoshi, M. (1981). Fertilization of the starfish *Astropecten aurantiacus*. *Exp. Cell Res.* **132,** 505–510.

Dangott, L. J., and Garbers, D. L. (1984). Identification and partial characterization of the receptor for speract. *J. Biol. Chem.* **259,** 13712–13716.

Dangott, L. J., Jordan, J. E., Bellet, R. A., and Garbers, D. L. (1989). Cloning of the mRNA for the protein that crosslinks to the egg peptide speract. *Proc. Natl. Acad. Sci. USA* **86,** 2128–2132.

Darszon, A., Labarca, P., Nishigaki, T., and Espinosa, F. (1999). Ion channels in sperm physiology. *Physiol. Rev.* **79,** 481–510.

Garbers, D. L. (1989). Molecular basis of fertilization. *Annu. Rev. Biochem.* **58,** 719–742.

Garbers, D. L., Watkins, H. D., Hansbrough, J. R., Smith, A., and Misono, K. (1982). The amino acid sequence and chemical synthesis of speract and of speract analogues. *J. Biol. Chem.* **257,** 2734–2737.

Heuser, J. E., and Salpeter, S. R. (1979). Organization of acetylcholine receptors in quick-frozen, deep-etched, and rotary-replicated *Torpedo* postsynaptic membrane. *J. Cell Biol.* **82,** 150–173

Hoshi, M., Nishigaki, T., Ushiyama, A., Okinaga, T., Chiba, K., and Matsumoto, M. (1994). Egg-jelly signal molecules for triggering the acrosome reaction in starfish spermatozoa. *Int. J. Dev. Biol.* **38,** 167–174

Ikadai, H., and Hoshi, M. (1981). Biochemical studies on the acrosome reaction of the starfish, *Asterias amurensis.* I. Factors participating in the acrosome reaction. *Dev. Growth Differ.* **23,** 73–80.

Kinoh, H., Shimizu, T., Fujimoto, H., and Suzuki, N. (1994). Expression of a putative precursor mRNA for sperm-activating peptide I in accessory cells of the ovary in the sea urchin *Hemicentrotus pulcherrimus. Roux's Arch. Dev. Biol.* **203,** 381–388.

Klotz, I. M. (1982). Numbers of receptor sites from Scatchard graphs: Facts and fantasies. *Science* **217,** 1247–1249.

Koyota, S., Wimalasiri, K. M. S., and Hoshi, M. (1997). Structure of the main saccharide chain in the acrosome reaction-inducing substance of the starfish, *Asterias amurensis. J. Biol. Chem.* **272,** 10372–10376.

Matsui, T., Nishiyama, I., Hino, A., and Hoshi, M. (1986). Induction of the acrosome reaction in starfish. *Dev. Growth Differ.* **28,** 339–348.

Matsumoto, M., Briones, A. V., Nishigaki, T., and Hoshi, M. (1999). Sequence analysis of cDNAs encoding precursors of starfish asterosaps. *Dev. Genet.* **25,** 130–136.

Nishigaki. T., Chiba, K., Miki, W., and Hoshi M. (1996). Structure and function of asterosaps, sperm-activating peptides from the jelly coat of starfish eggs. *Zygote* **4,** 237–245.

Ohtake, H. (1976). Respiratory behavior of sea-urchin spermatozoa. II. Sperm-activating substance obtained from jelly coat of sea-urchin eggs. *J. Exp. Zool.* **198,** 313–322.

Sase, I. Okinaga, T., Hoshi, M., Feigenson, G. W., and Kinosita, K. J. (1995). Regulatory mechanisms of the acrosome reaction revealed by multiview microscopy of single starfish sperm. *J. Cell Biol.* **131,** 963–973.

Scatchard, G. (1949). The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51,** 660–672.

Shimizu, T., Yoshino, K., and Suzuki, N. (1994). Identification and characterization of putative receptor for sperm-activating peptide I (SAP-I) in spermatozoa of sea urchin *Hemicentrotus pulcherrimus. Dev. Growth Differ.* **36,** 209–221.

Shimomura, H., Dangott, L. J., and Garbers, D. L. (1986). Covalent coupling of a resact analogue to guanylate cyclase. *J. Biol. Chem.* **261,** 15778–15782.

Singh, S., Lowe, D. G., Thorpe, D. S., Rodriguez, H., Kuang, W. J., Dangott, L. J., Chinkers, M., Goeddel, D. V., and Garbers, D. L. (1988). Membrane guanylate cyclase is cell-surface receptor with homology to protein kinases. *Nature* **334,** 708–712.

Smith, A. C., and Garbers, D. L. (1983). The binding of an ^{125}I-speract analogue to spermatozoa. *In* "Biochemistry of Metabolic Processes" (D. L. F. Lennon, F. W. Stratman, and R. N. Zahlten, Eds.), pp. 15–29. Elsevier, New York.

Suzuki, N., Nomura, K., Ohtake, H., and Isaka, S. (1981). Purification and the primary structure of sperm-activating peptides from the jelly coat of sea urchin eggs. *Biochem. Biophys. Res. Commun.* **99,** 1238–1244.

Suzuki, N., and Yoshino, K. (1992). The relationship between amino acid sequences of sperm-activating peptides and the taxonomy of echinoids. *Comp. Biochem. Physiol. B* **102,** 679–690.

Walter, B. D., Mao-Lin, H., Jacques, L., and Ramanath, Rao. (1981). Equilibrium and kinetic inhibition assays based upon fluorescence polarization. *Methods Enzymol.* **73,** 3–29.

Ward, G. E., Brokaw, C. J., Garbers, D. L., and Vacquier, V. D. (1985). Chemotaxis of *Arbacia punctulata* spermatozoa to resact, a peptide from the egg jelly layer. *J. Cell Biol.* **101,** 2324–2329.

Yamaguchi, M., Niwa, T., Kurita, M., and Suzuki, N. (1987). The participation of speract in the acrosome reaction of *Hemicentrotus pulcherrimus. Dev. Growth Differ.* **30,** 159–167.

Yoshino, K., and Suzuki N. (1992). Two classes of receptor specific for sperm-activating peptide III in sand-dollar spermatozoa. *Eur. J. Biochem.* **206,** 887–893.

Received for publication June 17, 1999
Revised November 5, 1999
Accepted December 13, 1999